Daughter of Sevenless Is a Substrate of the Phosphotyrosine Phosphatase Corkscrew and Functions during Sevenless Signaling

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Summary

The SH2 domain–containing phosphotyrosine phosphatase Corkscrew (CSW) is an essential component of the signaling pathway initiated by the activation of the *sevenless* receptor tyrosine kinase (SEV) during Drosophila eye development. We have used genetic and biochemical approaches to identify a substrate for CSW. Expression of a catalytically inactive CSW was used to trap CSW in a complex with a 115 kDa tyrosine-phosphorylated substrate. This substrate was purified and identified as the product of the *daughter of sevenless* (*dos*) gene. Mutations of *dos* were identified in a screen for dominant mutations which enhance the phenotype caused by overexpression of inactive CSW during photoreceptor development. Analysis of *dos* mutations indicates that DOS is a positive component of the SEV signaling pathway and suggests that DOS dephosphorylation by CSW may be a key event during signaling by SEV.

Introduction

Receptor tyrosine kinases (RTKs) play important roles in the regulation of cell growth and differentiation. The binding of a specific ligand to the extracellular domain of an RTK leads to receptor dimerization, activation of the cytoplasmic kinase domain, and autophosphorylation on tyrosine residues (Schlessinger and Ullrich, 1992). The activated receptor then initiates intracellular signaling pathways by interacting with specific cellular targets (Cantley et al., 1991). Frequently, these targets contain one or more SRC homology 2 (SH2) domains, which mediate their interactions with phosphorylated tyrosine residues of RTKs (Pawson, 1995). One crucial consequence of RTK activation is stimulation of RAS signaling activity (Egan and Weinberg, 1993). This stimulation depends on interaction of a complex, consisting of the adaptor molecule GRB2 and the guanine nucleotide exchange factor Son of sevenless (SOS), with the autophosphorylated receptor. Translocation of the GRB2–SOS complex from the cytosol to the plasma membrane results in an enhanced level of activated GTP bound RAS. Activated RAS, in turn, initiates a phosphorylation cascade consisting of the serine/threonine kinases RAF, MEK, and MAPK (Avruch et al., 1994). Activated MAPK can then modulate the activity of various transcription factors.

An extensively studied example of RTK mediated signal transduction is the role of the RTK encoded by the *sevenless* gene during Drosophila compound eye development (Zipursky and Rubin, 1994). The sole function of the *sevenless* RTK (SEV) is to trigger neuronal differentiation in the single R7 cell within each ommatidium of the eye in response to the presentation of its ligand, Bride of sevenless (BOSS), on the surface of the neighboring R8 photoreceptor cell. Using genetic screens and epistasis experiments, many components of the signal transduction pathway initiated in the R7 precursor cell by the activation of SEV have been identified. These include DRK (the Drosophila GRB2 homolog; Simon et al., 1993; Olivier et al., 1993), SOS (Simon et al., 1991; Bonfini et al., 1992), RAS1 (Simon et al., 1991), RAF (Dickson et al., 1992), MEK (Tsuda et al., 1993; Hsu and Perrimon, 1994), and MAPK (Brunner et al., 1994b; Biggs et al., 1994).

An unresolved question about signal transduction within the R7 cell is whether all the effects of SEV activation are mediated through the activation of RAS1 by DRK and SOS. Our previous studies have suggested the existence of a second signaling pathway whose function is required during SEV signaling (Allard et al., 1996). We identified dominantly inhibiting and constitutively signaling forms of corkscrew (CSW), a phosphotyrosine phosphatase (PTP) that contains two amino-terminal SH2 domains (Perkins et al., 1992). Previous studies have implicated CSW as an essential component of the signaling pathway initiated by the *torso* RTK (Perkins et al., 1992). We used these dominant *corkscrew* alleles to perform genetic epistasis experiments with activated and inhibiting forms of RAS1. The results indicated that efficient signaling by RAS1 and CSW are each dependent on the presence of the activity of the other. This suggested that CSW might act in a pathway that was parallel to the RAS1 pathway.

In this report, we have investigated the CSW signaling pathway by searching for substrates of the phosphatase activity of CSW. We describe the identification of a 115 kDa protein that is a CSW substrate and may also be a direct or indirect target for the tyrosine kinase activity of SEV. We show that this protein is encoded by the *daughter of sevenless* gene (*dos*; Raabe et al., 1996 [this issue of *Cell*]) and that mutations which inactivate *dos* dominantly enhance the effects of reduced CSW activity and suppress the effects of either excessive SEV or CSW activity. These results indicate that DOS is a crucial component of the SEV signaling pathway and strongly suggest that dephosphorylation of DOS by CSW promotes R7 photoreceptor differentiation.

*The first two authors contributed equally to this work.

Results

A 115 kDa Protein Is a Target of the PTP Activity of CSW

Our approach to understanding the role of CSW during SEV signaling was to identify physiological substrates of the phosphatase activity of CSW. The biochemical approach that we used was modeled after experiments which showed that a catalytically inactive mutant form of the MKP-1 phosphatase forms stable complexes in vivo with its substrates, phosphorylated p42^{mapk} and p44^{mapk} (Sun et al., 1993). We constructed an equivalently mutated form of *csw*, *csw*^{C583S}, and expressed the resulting protein (CSW^{CS}) in Drosophila SL2 cells under the control of the *Actin5C* promoter (Simon et al., 1989). The resulting cell line (SL2–CSW^{CS}) was examined for tyrosine-phosphorylated proteins that could be coprecipitated with CSW^{CS} using anti-CSW antisera. A 115 kDa phosphotyrosine-containing protein (p115) was specifically detected in CSW immunoprecipitates from lysates of SL2–CSW^{CS} cells but was barely detectable in anti-CSW immunoprecipitates of either untransfected SL2 cells or SL2 cells that overexpressed wild-type *csw* (SL2–CSW; Figure 1A). Antiphosphotyrosine immunoblotting of whole cell extracts from SL2–CSW^{CS} cells further indicated that p115 was one of the major tyrosine-phosphorylated proteins present in SL2–CSW^{CS} cells but was not a major phosphotyrosine-containing protein in SL2 or SL2–CSW cells (Figure 1B).

The most likely explanation for these results is that p115 is phosphorylated normally by endogenous kinases present in SL2 cells, but that SL2 cells contain active CSW, and perhaps other PTPs, which rapidly dephosphorylate p115. The expression of CSW^{CS} would then permit the accumulation of phosphorylated p115 by binding to and protecting p115 from the action of the endogenous CSW. A prediction of this model is that wild-type CSW must be capable both of forming a complex with phosphorylated p115 and of dephosphorylating p115. We tested each of these predictions. In order to demonstrate that phosphorylated p115 and wild-type CSW can associate in vivo, we examined whether phosphorylated p115 could be coprecipitated with wild-type CSW from normal SL2 cells which had been treated with the membrane-permeable PTP inhibitor pervanadate (Fantus et al., 1989; Heffetz et al., 1990). This treatment results in a rapid increase in the overall level of protein–tyrosine phosphorylation within the cells (data not shown). Tyrosine-phosphorylated p115 was readily detectable in anti-CSW immunoprecipitates from either SL2 or SL2–CSW^{CS} cells after treatment with pervanadate (Figure 1C). With increasing concentrations of the PTP inhibitor, the signal obtained in antiphosphotyrosine immunoblots was enhanced several-fold, and the p115 band showed decreased electrophoretic mobility, suggesting that p115 might be phosphorylated on multiple tyrosine residues under these conditions. Treatment with pervanadate also resulted in phosphorylation of CSW on tyrosine residues. In addition to p115, two other tyrosine-phosphorylated proteins with molecular masses of 170 kDa (p170) and 190 kDa (p190), respectively, could be coprecipitated both with CSW and CSW^{CS}. These results show that wild-type CSW can interact stably with phosphorylated p115, provided that CSW and possibly other PTPs are prevented from catalyzing the dephosphorylation of p115.

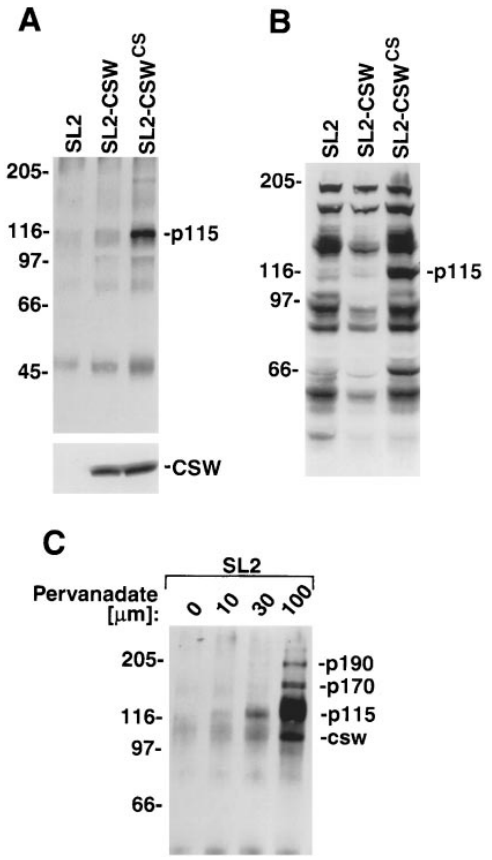

Figure 1. Interaction of CSW with a 115 kDa Tyrosine Phosphorylated Protein (p115)

The upper panel of (A) is an antiphosphotyrosine immunoblot of anti-CSW immunoprecipitates. The cell line used to prepare the extract is indicated above each lane. The lower panel of (A) is an anti-CSW immunoblot of the identical anti-CSW immunoprecipitates shown in the upper panel. Abundant CSW expression is seen in the SL2–CSW and SL2–CSW^{CS} lines. While not apparent in this exposure, SL2 cells also express a detectable amount of CSW.

(B) shows an antiphosphotyrosine immunoblot of total cell extracts from these same cell lines. The position of the tyrosine-phosphorylated p115 protein that coprecipitates with CSW^{CS} is indicated.

(C) shows an antiphosphotyrosine immunoblot of anti-CSW immunoprecipitates from SL2 cells that were treated with the PTP inhibitor pervanadate for 5 min prior to lysis. Similar results were obtained when SL2–CSW^{CS} cells were treated with pervanadate. The positions and molecular masses (kDa) of marker proteins are indicated to the left of each panel.

In order to demonstrate that CSW could use phosphorylated p115 as a substrate, tyrosine-phosphorylated p115 was isolated from pervanadate-treated SL2 cells by coprecipitation with anti-CSW antiserum. A purified bacterially expressed fusion protein (GST–CAT) that consisted of the CSW catalytic domain fused to glutathione S-transferase (GST) was then added to the immunoprecipitate, and the dephosphorylation of p115 was monitored by antiphosphotyrosine immunoblotting after SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The addition of GST–CAT resulted in the rapid dephosphorylation of p115 (Figure 2). In contrast, the addition

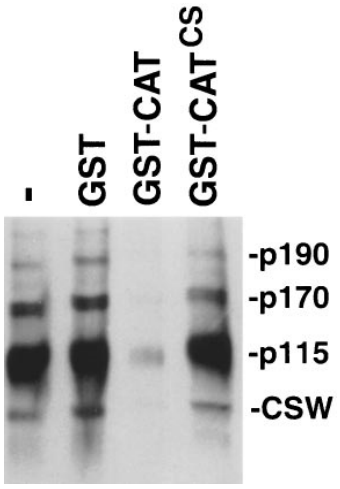

Figure 2. A Fusion Protein Containing the Catalytic Domain of CSW, GST–CAT, Can Dephosphorylate Tyrosine Phosphorylated p115

SL2 cells were treated with pervanadate, lysed, and then immunoprecipitated with anti-CSW antiserum. The washed precipitates were then incubated with either no added protein, GST, GST fused to the catalytic domain of CSW, or GST fused to an inactive CSW catalytic domain containing the *csw*^{C583S} mutation. Dephosphorylation of p115 was monitored by antiphosphotyrosine immunoblot. The positions of CSW and the p190, p170, and p115 proteins that precipitate with CSW from pervanadate-treated cells are indicated.

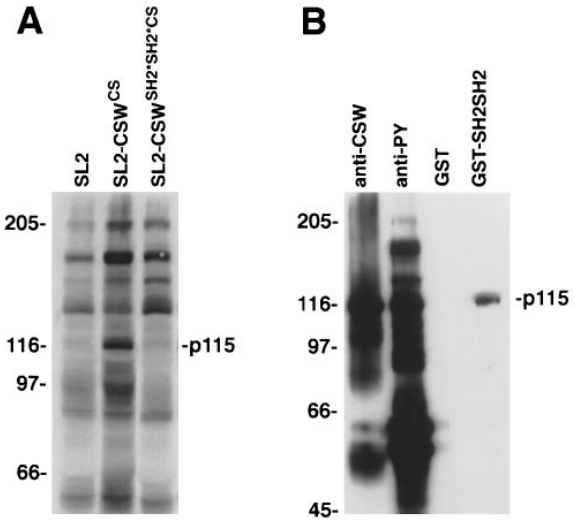

Figure 3. The SH2 Domains of CSW Are Crucial for Interacting with Tyrosine-Phosphorylated p115

(A) shows an antiphosphotyrosine immunoblot of whole cell extracts. The cell line used to prepare the extract is indicated above each lane.

(B) Binding of p115 to the CSW SH2 domains. In order to remove excess CSW^{CS} that was not bound to p115, the CSW^{CS}/p115 complex was partially purified from SL2–CSW^{CS} cells by ion-exchange chromatography. The column eluate was subjected to precipitation with either anti-CSW antiserum, antiphosphotyrosine antibodies, GST bound to glutathione–agarose beads, or GST–SH2SH2 bound to glutathione–agarose beads. The presence of p115 was detected by antiphosphotyrosine immunoblot. Despite the presence of numerous phosphotyrosine-containing proteins in the sample, only p115 could be precipitated by the SH2 domains of CSW.

of GST alone or a GST–CAT protein containing the inactivating *csw*^{C583S} substitution (GST–CAT^{CS}) had no effect on the phosphotyrosine content of p115. These results indicate that CSW can dephosphorylate p115 in vitro and support the proposal that phosphorylated p115 is a direct target for CSW dephosphorylation. Tyrosine-phosphorylated p170 and p190 were also rapidly dephosphorylated by the addition of GST–CAT, indicating that these proteins might also be possible substrates of CSW.

The Interaction Between p115 and CSW^{CS} Depends on the SH2 Domains of CSW

The presence of phosphorylated tyrosine residues on p115 suggested that the association of CSW and p115 might involve interactions between phosphorylated p115 and one or both of the SH2 domains of CSW. In order to address this issue, we established an SL2 cell line, which stably expressed CSW^{CS}, that contained inactivating mutations in both of the SH2 domains (CSW^{SH2*SH2*CS}). Residues arginine-32 and arginine-137 were exchanged for lysine residues. These residues are part of the highly conserved FLVRES motif and correspond to arginine-175 of c-src, a residue which is critical for interaction of the SH2 domain with tyrosine-phosphorylated ligands (Pawson, 1995). Lysates of this cell line were analyzed for the presence of tyrosine-phosphorylated p115 (Figure 3A). The expression of CSW^{SH2*SH2*CS} was not sufficient to induce an accumulation of tyrosine-phosphorylated p115. These results suggest that one or both of the SH2 domains of CSW are important for the interaction of CSW with phosphorylated p115.

The ability of the SH2 domains of CSW to interact with phosphorylated p115 was confirmed by demonstrating that an immobilized fusion protein containing GST and the SH2 domains of CSW (GST–SH2SH2) was able to precipitate phosphorylated p115 from extracts of SL2–CSW^{CS} cells. In order to generate conditions in which GST–SH2SH2 could effectively compete with CSW^{CS} for binding to phosphorylated p115, the bulk of free CSW^{CS} was removed from the extracts of SL2–CSW^{CS} cells by anion exchange chromatography. After removal of free CSW^{CS}, GST–SH2SH2 was able effectively to precipitate phosphorylated p115 (Figure 3B). Despite the presence of many tyrosine-phosphorylated proteins in the eluate, p115 was the only tyrosine-phosphorylated protein that bound to GST–SH2SH2, indicating the high degree of specificity in the interaction of tyrosine-phosphorylated p115 and the SH2 domains of CSW. These results confirm that the SH2 domains of CSW play an important role in p115 recognition.

p115 Is Tyrosine-Phosphorylated in Response to Constitutively Active SEV

Since SL2 cells do not express SEV, the tyrosine phosphorylation of p115 in SL2–CSW^{CS} cells must be catalyzed by other kinases that are present in these cells. However, the crucial role of CSW during SEV signaling suggested that p115 might also be a substrate for SEV. In order to examine the effect of SEV activity on p115

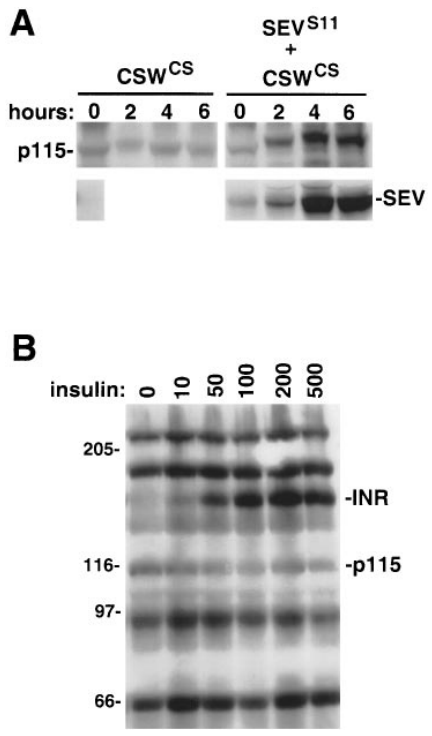

Figure 4. Induction of SEV But Not Insulin Receptor Kinase Activity Leads to Increased Phosphorylation of p115

The upper portion of (A) shows an antiphosphotyrosine immunoblot of extracts prepared from either SL2–CSW^{CS} or SL2–CSW^{CS} cells that express SEV^{S11} under the control of a heat-shock responsive promoter. Cells either were not heat shocked or were placed at 37°C for 30 min and then allowed to recover for 2, 4, or 6 hr. The lower panel of (A) shows an anti-SEV immunoblot of the same extracts that shows the accumulation of the 58 kDa subunit of SEV. Induction of SEV activity leads to an increase in the intensity of antiphosphotyrosine staining of p115 and a shift of p115 to a larger apparent molecular mass. In contrast, heat-shock treatment of SL2–CSW^{CS} cells leads only to a much more transient change in electrophoretic mobility and no apparent change in the phosphotyrosine content of p115. (B) shows an antiphosphotyrosine immunoblot of extracts prepared from SL2–CSW^{CS} cells that were serum-starved for 24 hr and then treated with various concentrations of bovine insulin. The concentration of insulin (ng/ml) used is given above each lane. Addition of insulin results in significant activation and phosphorylation of insulin receptor without any observable change in the phosphorylation or electrophoretic mobility of p115.

phosphorylation, we expressed a constitutively activated version of SEV (SEV^{S11}) under heat-shock promoter control in SL2 cells (SL2–SEV^{S11} cells) or in SL2 cells that also expressed CSW^{CS} (SL2–CSW^{CS}SEV^{S11} cells; Basler et al., 1991; Simon et al., 1993). Upon heat-shock induction, SEV^{S11} is synthesized as a 90 kDa precursor, which is then cleaved to yield 58 and 48 kDa forms (Simon et al., 1991). While SEV^{S11} autophosphorylation could be readily detected in SL2–SEV^{S11} cells, SEV^{S11} expression did not result in other obvious changes in the spectrum of cellular tyrosine phosphorylation (data not shown). In contrast, induction of SEV^{S11} in cells expressing CSW^{CS} led to an increase in the tyrosine-phosphate content and a concurrent decrease in the electrophoretic mobility of p115 (Figure 4A). Thus, the induction of SEV activity can lead either directly or indirectly to enhanced tyrosine phosphorylation of p115. Furthermore, the apparent shift in mobility upon SEV^{S11} induction suggested that at least some of the induced p115 phosphorylation occurred at sites that were distinct from those sites which were phosphorylated in SL2–CSW^{CS} cells that did not express SEV. As a control for the effects of heat shock on p115 phosphorylation, we also examined the tyrosine phosphorylation and mobility of p115 from heat-shocked SL2–CSW^{CS} cells. In this case, no increase in tyrosine-phosphate content was observed, though there was a short-lived decrease in electrophoretic mobility that might be due to other covalent modifications of p115, such as serine/threonine phosphorylation.

These results suggest that p115 is either a direct or indirect target for the kinase activity of SEV. One issue that is raised by these results is whether p115 might also be a target for phosphorylation in response to the induction of other RTKs. Therefore, we examined the effect on p115 phosphorylation of activation of the Drosophila insulin receptor in response to mammalian insulin (Fernandez et al., 1995). SL2 and SL2–CSW^{CS} cells were each starved for 24 hr in serum-free media and then stimulated with increasing concentrations of insulin. Antiphosphotyrosine immunoblot analysis revealed a dose-dependent increase in tyrosine phosphorylation of a 170 kDa protein that most likely represented the insulin receptor according to its response to insulin and molecular mass (Figure 4B). Insulin stimulation had no effect on the phosphorylation state of p115. This result indicates that the ability of SEV^{S11} to induce tyrosine phosphorylation of p115 is not a general property possessed by all RTKs.

Purification of p115

The evidence that p115 is both a substrate for dephosphorylation by CSW and a target for either direct or indirect phosphorylation by SEV indicated that p115 might play a crucial role in signaling by SEV. We sought to investigate this role both by identifying the gene that encoded p115 and by studying mutations that inactivated p115. In order to identify the p115 gene, we purified sufficient p115 for microsequence analysis. The purification scheme was based on the ability of CSW^{CS} to bind phosphorylated p115 specifically and to prevent dephosphorylation by cellular phosphatases including CSW. Cytosolic extracts of SL2–CSW^{CS} cells were prepared in the absence of PTP inhibitors and allowed to undergo dephosphorylation by endogenous PTPs for 50 min at room temperature. During this period, virtually all phosphotyrosine-containing proteins in the extract were dephosphorylated, with the exception of p115. Following this treatment, the extract was denatured by boiling in an SDS-containing buffer, diluted to lower the concentration of SDS, and then immunoprecipitated with antiphosphotyrosine antibodies. This procedure resulted in a preparation that contained a single major protein band that was 115 kDa in apparent molecular mass. We used this protocol to purify approximately 4 μg of p115 from 4×10^9 SL2–CSW^{CS} cells. The sequence of a single tryptic peptide (XXMSVGNLYSQGXN) was obtained.

A Screen for Mutations Affecting CSW Function

At the same time that we sought to identify the gene that encoded p115, we also sought to isolate mutant alleles of the gene encoding p115 and of other genes that might encode components of the CSW portion of the SEV signaling pathway. We began by examining the effects of expression of CSW^{CS} in the developing eye. For this purpose, a P element, P[*sevhs–csw*^{CS}], which expressed CSW^{CS} under the control of *sevenless* transcriptional enhancer elements, was constructed and transformed into flies. This P element directed expression of CSW^{CS} in several cells of the developing eye disc, including the precursors to photoreceptors R3, R4, and R7. Flies carrying P[*sevhs–csw*^{CS}] had mildly roughened eyes owing to the frequent absence of photoreceptors R3, R4, and R7 from mature ommatidia (Figures 5A and 5B). This phenotype is the result of the ability of CSW^{CS} to inhibit the function of wild-type CSW, which is required for neuronal differentiation in these cells (Allard et al., 1996). The inhibitory effect of CSW^{CS} expression could be entirely reversed by the expression of wild-type CSW (data not shown).

We reasoned that if p115 was an important target of CSW during photoreceptor development, then lowering the amount of functional p115 present during photoreceptor development by inactivating one copy of the p115 gene might affect the severity of the phenotype induced by P[*sevhs–csw*^{CS}]. However, since we did not know whether p115 might need to be dephosphorylated to allow its active participation in signaling, or whether dephosphorylation might instead prevent phosphorylated p115 from blocking signaling, we could not predict whether p115 mutations would make the P[*sevhs–csw*^{CS}] phenotype more or less severe. We therefore mutagenized flies and screened for mutations that acted either as dominant enhancers or suppressors of P[*sevhs–csw*^{CS}]. The complete results of the mutagenesis screen will be published elsewhere. In summary, approximately 75,000 progeny of ethylmethylsulfonate-treated flies were screened. Mutations were identified which affected several genes that were already known to encode important components of the SEV signaling pathway. Suppressors of P[*sevhs–csw*^{CS}] included activated alleles of *Sos*. Inactivating alleles of *sos*, *Ras1*, and *pointed* were identified as enhancers of the P[*sevhs–csw*^{CS}] phenotype (Simon et al., 1991; O'Neill et al., 1994; Brunner et al., 1994a).

The screen for mutations that modify the P[*sevhs–csw*^{CS}] phenotype also identified several additional genes that have not been previously described. We chose to focus on one of the novel loci, *E(csw*^{CS}*)3A*, for several reasons. First, we isolated seven alleles of *E(csw*^{CS}*)3A*. This high frequency indicated that these alleles were likely to result from inactivation of the *E(csw*^{CS}*)3A* gene rather than from the creation of a novel function. Second, the effect of *E(csw*^{CS}*)3A* mutations on the P[*sevhs–csw*^{CS}] phenotype (Figures 5C and 5D) was stronger than the effects of mutations in any of the other novel complementation groups that we identified. Since our data suggested that p115 was the direct target for CSW action, we expected that mutations in the p115 gene would have a profound effect on the P[*sevhs–csw*^{CS}] phenotype. Third, *E(csw*^{CS}*)3A* mutations are capable of acting as dominant suppressors of the ability of a membrane-targeted form of CSW (CSW^{src90}) to induce R7 development in the cone cell precursors (Figures 5D and 5H; Allard et al., 1996). This result was consistent with the *E(csw*^{CS}*)3A* product playing a crucial role downstream of CSW during R7 development. Finally, the phenotype of clones of homozygous mutant *E(csw*^{CS}*)3A* cells generated in the eyes of heterozygous animals was identical to the clonal phenotype of cells lacking *csw* function (Allard et al., 1996). In each case, homozygous mutant cells can proliferate but do not differentiate as photoreceptors (data not shown; Allard et al., 1996). These results indicate that the *E(csw*^{CS}*)3A* product, like CSW, is required during the development of every photoreceptor of the developing ommatidium.

In order to understand more about where the *E(csw*^{CS}*)3A* protein might act during SEV signaling, we examined the effects of *E(csw*^{CS}*)3A* mutations on signaling by activated SEV (SEV^{S11}), RAS1 ($RAS1^{V12}$), and RAF (RAF^{TOR}) proteins expressed under the control of *sevenless* transcriptional enhancers (Basler et al., 1991; Fortini et al., 1992; Dickson et al., 1992). The external eye morphology is a sensitive assay for limiting components of the signaling pathway that are downstream of these activated proteins, because expression of the activated proteins results in a dose-dependent eye roughness owing to the abnormal recruitment of cone cell precursors to the R7 developmental fate (Dickson et al., 1995; Chang et al., 1995; Therrien et al., 1995). Heterozygosity for *E(csw*^{CS}*)3A* suppressed the phenotype caused by SEV^{S11} but had no effect on the phenotype caused by $RAS1^{V12}$ or RAF^{TOR} (Figure 5). These results indicated that the level of *E(csw*^{CS}*)3A* function was limiting during signaling by activated SEV, but not during signaling by either activated RAS1 or RAF. These data are consistent with placement of the *E(csw*^{CS}*)3A* protein on a part of the SEV signaling pathway that is either before RAS1/RAF activation or in a pathway that is parallel to the activation of RAS1 and RAF.

E(csw^{CS}*)3A* Is *dos* and Encodes p115

The *E(csw*^{CS}*)3A* locus was meiotically mapped to the 62E–F region of the Drosophila chromosomes. We then became aware that a screen conducted in the laboratory of E. Hafen, for mutations that dominantly suppress the SEV^{S11} phenotype, had identified a gene, *dos*, that resided in this same region of the chromosome (Raabe et al., 1996). Since both *dos* and *E(csw*^{CS}*)3A* mutations are lethal when homozygous, we tested for allelism by asking whether *E(csw*^{CS}*)3A* and *dos* mutations complemented each other for viability. The mutations failed to complement, indicating that *E(csw*^{CS}*)3A* is *dos*.

Raabe et al. (1996) had already isolated the *dos* gene and determined its sequence. Conceptual translation of the *dos* cDNA predicts a protein of 97 kDa that has little homology to known proteins except for the presence of a pleckstrin homology domain (see Raabe et al., 1996, for a full discussion of the *dos* sequence; Gibson et al., 1994). However, immunoblotting with antibodies to DOS indicates that DOS migrates as a 115 kDa protein in SDS–PAGE (T. R. and E. Hafen, unpublished data). We

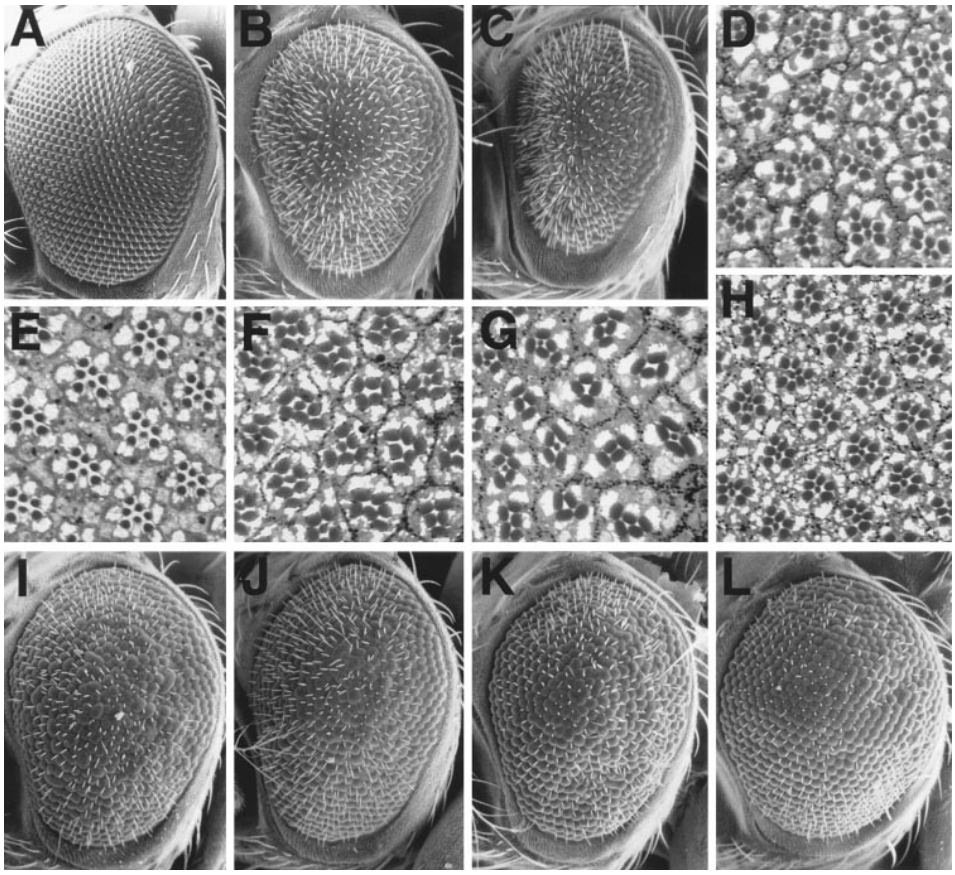

Figure 5. Genetic Analyis of *E(csw^{CS})3A* Mutations

(A) shows a scanning electron micrograph (SEM) of a phenotypically wild-type eye of a *w^{1118}* fly. The eye is smooth in appearance and has the normal regular array of photoreceptors. This can be seen in apical tangential sections of *w^{1118}* flies (E). The pattern of seven photoreceptors can be seen by observing the darkly staining structures that are the rhabdomeres (light-sensing organelles) of each photoreceptor. The presence of R7 cells can be scored by the presence of the R7 rhabdomere, which is smaller and more centrally located than the rhabdomeres of R1–R6.

(B) shows an SEM of an eye of a heterozygous P[*sevhs–csw^{CS}*] fly. The surface of the eye is roughened owing to abnormalities in the structure of the underlying ommatidia (F), including the frequent absence of R7 and one or two of the R1–R6 class photoreceptors. Examination of the neuronal development of these eyes indicates that R3 and R4 are most frequently absent (data not shown).

(C) shows an SEM of a fly that is heterozygous both for P[*sevhs–csw^{CS}*] and for *E(csw^{CS})3A$^{1.46}$*. The presence of the *E(csw^{CS})3A$^{1.46}$* mutation enhances the eye roughness of flies carrying P[*sevhs–csw^{CS}*] and leads to more severe abnormalities in the photoreceptor content of individual ommatidia (G). Flies heterozygous for the *E(csw^{CS})3A* mutations alone have no detectable defects in eye morphology or ommatidial structure (data not shown).

(D) shows an apical tangential section of P[*sevhs–csw^{src90}*] flies. Many ommatidia have extra R7 photoreceptors owing to enhanced signaling by the membrane-targeted CSW^{src90} protein.

(H) The dominant effect of enhanced signaling by CSW^{src90} is suppressed by the *E(csw^{CS})3A$^{2.42}$* mutation. The average number of R7-like cells per ommatidium is reduced from 2.15 to 1.4 by the presence of the *E(csw^{CS})3A$^{2.42}$* mutation. This result suggests that the *E(csw^{CS})3A* protein is a limiting component during CSW^{src90} signaling.

(I) and (J) show SEMs of flies heterozygous for P[*sevRas1^{V12}*]2 or both P[*sevRas1^{V12}*]2 and *E(csw^{CS})3A$^{1.46}$*, respectively (Fortini et al., 1992). The presence of the *E(csw^{CS})3A* mutation has no apparent effect on the signaling ability of RAS1^{V12}. Similar results were obtained with flies expressing the activated RAF^{Tor} protein instead of RAS1^{V12} (data not shown).

(K) and (L) show SEMs of flies hemizygous for P[*SE–Sev^{S11}*] (K) or hemizygous for P[*SE–Sev^{S11}*] and heterozygous for *E(csw^{CS})3A$^{1.46}$* (L). In contrast to signaling by RAS1^{V12}, the effectiveness of signaling by SEV^{S11} was attenuated by the presence of the *E(csw^{CS})3A$^{1.46}$* mutation, indicating that the *E(csw^{CS})3A* protein is a limiting component during SEV^{S11} signaling. Similar results for all these experiments were obtained with several alleles of *E(csw^{CS})3A*.

therefore directly tested whether *dos* encoded p115 by searching for the p115 peptide sequence (XXMSV GNLYSQGXN) in DOS. A match was found in the predicted DOS tryptic fragment SSMSVGNLYSQGGN-GASGMR (amino acids 816–835). This indicated that *dos* encoded the p115 substrate of CSW. In order to validate this conclusion further, we performed anti-DOS immunoblot analysis, both of anti-CSW immunoprecipitates from SL2–CSW^{CS} cells and of purified p115 preparations (Figure 6). These results clearly show that the tyrosine-phosphorylated p115 protein that coprecipitated with CSW^{CS} and the protein that we purified for micro-sequence analysis are recognized by anti-DOS antibodies.

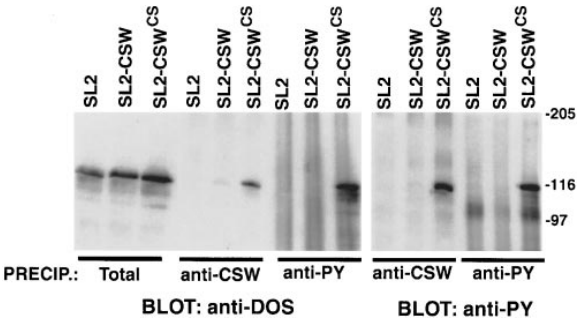

Figure 6. The Tyrosine-Phosphorylated p115 Protein Is Recognized by Anti-DOS Antibodies

Cytosolic extracts were prepared from the cell line indicated above each lane. These extracts were then either precipitated with anti-CSW antiserum and subjected to SDS–PAGE (anti-CSW), or else first allowed to undergo dephosphorylation by endogenous PTPs and then denatured and precipitated with antiphosphotyrosine antibodies and subjected to SDS–PAGE (anti-PY). The procedure used to generate the protein samples in the anti-PY precipitates is equivalent to that used to generate p115 for microsequencing and yields a major 115 kDa band from SL2–CSW^{CS} cells but not from either SL2 or SL2–CSW cells. The right panel shows an antiphosphotyrosine immunoblot of these samples and shows that tyrosine-phosphorylated p115 is present at high levels in the anti-CSW and anti-PY samples from SL2–CSW^{CS} cells but not from equivalent samples of either SL2 or SL2–CSW cells. The left panel shows an anti-DOS immunoblot of the same precipitates and of unprecipitated extracts (Total). Analysis of the unprecipitated extracts shows that DOS is present in extracts of all three cell lines and that DOS migrates as a 115 kDa protein during SDS–PAGE. Analysis of the anti-CSW samples indicates that DOS coprecipitates with CSW^{CS} but not with wild-type CSW produced in either SL2 or SL2–CSW cells. Similarly, DOS is found in the anti-PY precipitates of SL2–CSW^{CS} cells but not in those derived from either SL2 or SL2–CSW cells.

Discussion

During Drosophila ommatidial development, effective signaling by the SEV RTK is essential for the precursors of R7 photoreceptor cells to differentiate as neurons. Previous studies have provided evidence for the possible existence of two signaling pathways that are crucial for signaling by SEV. The better characterized pathway is initiated by recruitment of the SOS–DRK signaling complex to the activated and autophosphorylated SEV (Zipursky and Rubin, 1994). This recruitment step leads to the activation of RAS1 and the stimulation of a MAPK pathway that is very similar to those that have been characterized in vertebrate cells. The less characterized pathway involves the action of the SH2 domain–containing PTP CSW. Our previous genetic studies of dominant *csw* alleles have indicated that CSW function is essential for SEV action and have further suggested that at least a portion of CSW function is either downstream of RAS1 activation or else in a pathway that acts in parallel to the RAS1/RAF pathway (Allard et al., 1996). In this study, we have further characterized the action of CSW during SEV signaling by identifying a substrate for the PTP activity of CSW.

In our biochemical search for CSW substrates, we employed a catalytically inactive CSW, CSW^{CS}, to trap a substrate in a complex with CSW. We found that overexpression of CSW^{CS} in Drosophila SL2 cells resulted in the formation of a complex between CSW^{CS} and phosphotyrosine-containing protein, DOS. Several lines of biochemical evidence suggest that DOS is a genuine substrate for CSW. First, previous studies of other PTPs in which the equivalent cysteine residue has been mutated to serine suggest that this mutation creates a catalytic domain that is inactive but that retains the ability to bind substrate with high affinity (Sun et al., 1993; Milarski et al., 1993; Guan and Dixon, 1991). Second, the addition of a fusion protein containing a catalytically active domain of CSW to extracts containing tyrosine-phosphorylated DOS leads to the rapid dephosphorylation of DOS. Finally, the ability of the CSW^{CS} protein to protect DOS from dephosphorylation by other PTPs during our DOS purification protocol suggests that direct contacts are made between the catalytic domain of CSW and the phosphorylated tyrosine residues of DOS. In addition, the ability of DOS mutations to act as genetic modifiers of the CSW^{CS} rough-eye phenotype is entirely consistent with DOS being a direct target of CSW.

Models for CSW and DOS Function During SEV Signaling

Our isolation of *dos* mutations as dominant enhancers of the phenotypic effects of inadequate CSW function implies that DOS cooperates with CSW during SEV signaling. When combined with the ability of DOS to act as a substrate for the PTP activity of CSW, this genetic information suggests possible roles for CSW and DOS during SEV signaling. We propose that prior to SEV activation, DOS phosphorylation at specific tyrosine residues is maintained by the action of a presently unidentified kinase. The activation of SEV by BOSS then leads to activation of the PTP activity of CSW and the subsequent dephosphorylation of DOS by CSW. Dephosphorylated DOS would then be free to participate actively in the initiation of R7 photoreceptor development. Proof of this model for CSW and DOS function will require identification of the particular phosphotyrosine residues of DOS which are targets for dephosphorylation by CSW. This model predicts that mutations which reduce the phosphorylation of these specific tyrosines might generate overly active DOS.

Since the only recognizable feature of the predicted DOS peptide sequence is the presence of a pleckstrin homology domain, we presently can only speculate on the precise role of DOS function after dephosphorylation by CSW during SEV signaling (Raabe et al., 1996). The ability of DOS mutations to act as dominant suppressors of the phenotype caused by expression of an activated SEV but not the same phenotype when induced by activated RAS1 or RAF expression suggests two possible placements of DOS action in the SEV signaling pathway. The simplest model is that the sole important function of DOS is to contribute to RAS1 activation (Figure 7A). DOS mutations would thus fail to act as heterozygous suppressors of RAS1^{V12} signaling because the RAS1^{V12} protein would already be fully activated. The second possibility is that DOS functions in a pathway that normally acts in conjunction with RAS1 during SEV signaling (Figure 7B). This model postulates that SEV normally

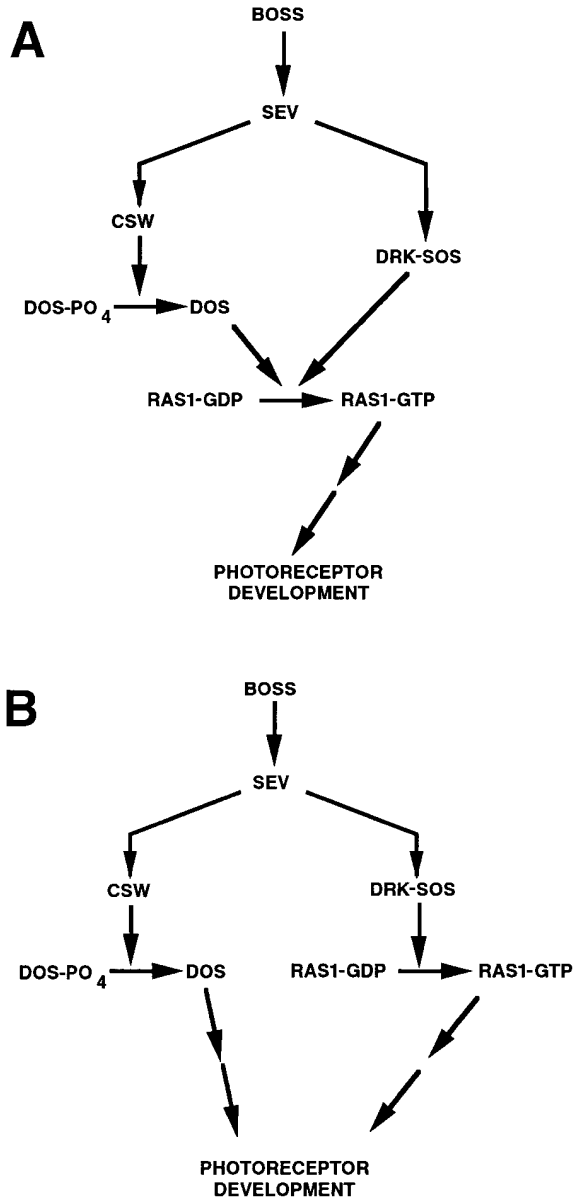

Figure 7. Two Models for the Action of DOS during R7 Differentiation

Only the phosphorylated tyrosine residue or residues of DOS which are a substrate for dephosphorylation by CSW are indicated. Other tyrosine residues which may be sites for phosphorylation by SEV are not shown. See Discussion for details.

directs R7 development by combining a moderate level of RAS1 activation with the stimulation of other positive signaling molecules such as DOS and CSW, but that overexpression of mutationally activated RAS1 or RAF activates the MAPK cascade so strongly that activation of the other branches of the SEV signaling pathway is no longer required. Under these conditions, the reduction of function of the CSW–DOS signaling branch caused by the inactivation of only one allele of DOS might have little effect on signaling. Thus, DOS mutations would fail to act as dominant suppressors of the activated RAS1 and RAF phenotypes. It is worth noting that this model does not exclude the possibility that DOS and CSW also contribute to RAS1 activation. Each of these models is entirely consistent with presently available genetic data. Support for the first model comes from several studies of CSW and its mammalian homolog, SH–PTP2, which have suggested that the level of CSW function can affect RAS1 and MAPK activation (Lu et al., 1993; Li et al., 1994; Bennett et al., 1994; Milarski and Saltiel, 1994; Noguchi et al., 1994; Tang et al., 1995; Zhao et al., 1995). Support for the second model comes both from numerous biochemical studies of vertebrate RTK signaling which have demonstrated that RTKs stimulate multiple signaling pathways and from our previous genetic experiments, which have shown that CSW function during R7 development cannot be placed genetically solely upstream of RAS1 activation (Allard et al., 1996). A fuller understanding of the role of DOS and CSW will require elucidation of the activity of DOS and the targets of DOS action.

These models are each consistent with all of our present data, but both leave many issues unresolved. One important question is the identity and possible regulation of the kinase that maintains DOS in a phosphorylated state prior to the activation of SEV. Since this phosphorylation is proposed to maintain DOS in an inactive state, we predict that mutations which decrease the activity of this kinase might be expected to act as suppressors of the phenotype caused by CSW^{CS} expression. Several new suppressor loci were found in our genetic screen for CSW^{CS} modifiers, but the products of these genes have not yet been characterized.

Another critical issue raised by our proposed models is how CSW activity might be regulated by SEV. It is important to note that none of our present genetic data demands that the level of CSW activity change during SEV signaling. Another possibility is that CSW maintains DOS in its dephosphorylated state at all times. Our data suggest that this may be the case in SL2 cells, because phosphorylation of DOS is only detectable in the presence of CSW^{CS}. Nevertheless, regulation of CSW by SEV is an attractive proposal, because RTK induced activation of the mammalian homolog of CSW, SH–PTP2, has been previously reported (Vogel et al., 1993; Lechleider et al., 1993). SH–PTP2 activation appears to be mediated by binding of the SH2 domains of SH–PTP2 to specific phosphorylated tyrosines of the activated RTK (Vogel et al., 1993; Feng et al., 1993; Lechleider et al., 1993). In some cases, phosphorylation of SH–PTP2 by the activated RTK also occurs and may contribute to the enhancement of SH–PTP2 activity (Vogel et al., 1993; Feng et al., 1993; Lechleider et al., 1993). This model for PTP activation is unlikely to apply to the interaction of CSW with SEV. While we have detected coimmunoprecipitation of CSW and SEV, this interaction is dependent neither on SEV phosphorylation nor on functional SH2 domains of CSW (unpublished data). Furthermore, SEV appears to be unable to induce CSW phosphorylation in SL2 cells. Instead, our results suggest that the primary function of the SH2 domains of CSW during SEV signaling may be the recognition of DOS.

The role of DOS phosphorylation by SEV is another important issue that will require further study. Our current results indicate that SEV activation can lead to enhanced DOS phosphorylation in cultured Drosophila

cells, but we do not have any direct evidence for SEV phosphorylation of DOS in the R7 precursor cell during SEV signaling. Several roles for this putative phosphorylation of DOS by SEV seem possible. DOS phosphorylation by SEV could act to enhance the ability of the SH2 domains of CSW to bind to DOS and thus be a mechanism by which SEV stimulates CSW to dephosphorylate key phosphotyrosines of DOS. Alternatively, phosphorylation of DOS by SEV could stimulate a presently unknown catalytic activity of DOS. Finally, phosphorylation of DOS might allow DOS to bind to other signaling molecules in a manner similar to IRS-1 and GAB-1 (Myers and Morris, 1993; Hogado-Madruga et al., 1996). Interestingly, each of these proteins shares with DOS the property of having an amino-terminal pleckstrin homology domain (Gibson et al., 1994).

Conservation with Other RTK Signaling Pathways

Since signaling pathways of Drosophila and mammalian RTKs are highly conserved, it is likely that a vertebrate homolog of p115 exists which is a substrate of SH–PTP2. Two potential SH–PTP2 substrates that are approximately the same size as DOS have been described. A 115 kDa phosphotyrosine protein could be coprecipitated with either wild-type or catalytically inactive SH–PTP2 from insulin-treated cells (Yamauchi et al., 1995). This protein has several properties that are distinct from DOS. For example, this p115 could not be precipitated by the SH2 domains of SH–PTP2 and was tyrosine-phosphorylated in response to insulin treatment. Another study detected a 120 kDa tyrosine-phosphorylated protein whose properties are similar to the Drosophila p115 (Milarski and Saltiel, 1994). The 120 kDa protein was only tyrosine-phosphorylated in cells expressing an inactive SH–PTP2 and could be precipitated by the SH2 domains of SH–PTP2. Determining if one or more of these proteins is homologous to DOS will require the cloning of their respective cDNAs. An interesting possibility is that both CSW and SH–PTP2 may each have a number of different substrates in different types of cells and in response to the activation of different RTKs.

Experimental Procedures

Drosophila Culture and Histology

Fly culture, crosses, mutagenesis, and germline transformation were performed using standard procedures. Mutagenized males from an isogenic stock were crossed to females of the genotype TM3, P[*sevhs–csw*^{cs}]/TM6b. F1 progeny were scored for modifications in the rough-eye phenotype caused by the expression of CSW^{cs}. Histological analysis of flies was as previously described (Simon et al., 1991). The Drosophila Schneider cell line 2 (SL2) was maintained and transfected as previously described (Simon et al., 1989). Selection of polyclonal cell lines was in G418 (1 mg/ml) or hygromycin B (200 μg/ml). For heat-shock induction of SEV^{S11}, cells were treated for 30 min at 37°C and then returned to 23°C.

Molecular Biology

Mutants in *csw* were generated as previously described (Allard et al., 1996). For expression of wild-type and mutant versions of CSW, all constructs were subcloned as *KpnI* fragments into an expression vector pAT-Hygro, which uses an *Actin5C* promoter to drive expression. The SEV^{S11} expression constructs were described previously (Simon et al., 1993). The *csw*^{CS} coding sequence was cloned into the *sev* enhancer containing P element vector pKB267 for germline transformation of flies (Basler et al., 1991).

Protein Analysis

Immunoblotting and immunoprecipitation were prepared as previously described (Herbst et al., 1995). The following antibodies were used: PY20, a monoclonal antibody against phosphotyrosine (Transduction Laboratories); monoclonal antibody 78C10, which recognizes the catalytic domain of SEV; CT-1, a rat polyclonal antiserum raised against a GST–fusion protein containing the carboxy-terminal 159 amino acids of CSW; CT-1–2, a polyclonal rabbit antiserum raised against a GST–fusion protein containing the carboxy-terminal 610 amino acids, and affinity purified with the antigen used to raise CT-1. The serum CT-1 was used for all immunoprecipitations of CSW, while CT-1–2 was used for immunoblotting. Anti-DOS antibodies are described by Raabe et al. (1996). The GST expression vectors used were either pGEX (Guan and Dixon, 1991) or pGEX-2TK (Pharmacia).

For in vitro dephosphorylation experiments, the catalytic domain of CSW (residues 231–681) was expressed as a GST–fusion protein (GST–CAT). SL2 cells were incubated with 200 μM pervanadate for 15 min at room temperature, lysed in lysis buffer without phosphatase inhibitors, and immunoprecipitated (10^7 cell equivalents) with antiserum CT-1. The precipitates were first washed twice with washing buffer (without phosphatase inhibitors) and then once in phosphatase buffer (100 mM MES [pH 6.8], 150 mM NaCl, 5 mM DTT, and 2 mM EDTA). Dephosphorylation reactions were done by resuspending the precipitates in 50 μl of phosphatase buffer containing 3 μg of GST–fusion proteins and incubating for 30 min at room temperature. The reactions were stopped by the addition of SDS sample buffer.

Ion exchange chromatography of CSW–DOS was performed as follows. Extracts from SL2–CSW^{CS} cells were prepared by three freeze/thaw cycles in buffer A (20 mM Tris [pH 7.5], 10% glycerol, 2 mM orthovanadate, 10 mM NaF, 2 mM sodium pyrophosphate) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin, cleared by centrifugation at 12,500 × g for 20 min at 4°C, diluted with an equal volume of buffer A/200 mM NaCl, and loaded on a High-Q anion exchange column (BioRad). The column was washed with buffer A/100 mM NaCl and bound proteins eluted with buffer A/200 mM NaCl. For precipitations with GST–SH2SH2 (amino acids 1–218 of CSW), the eluate was adjusted to 150 mM NaCl and 0.5% Triton X-100 and mixed with glutathione beads to which GST–SH2SH2 was bound.

For purification of DOS, SL2–CSW^{CS} cells (4×10^9) were harvested by centrifugation, lysed in 20 ml of lysis buffer lacking phosphatase inhibitors, incubated at room temperature for 50 min, and then centrifuged at 45,000 × g for 30 min at 4°C. The supernatant was then precleared by incubation with agarose cross-linked to monoclonal antibody PY20 for 1 hr at 4°C, adjusted to 1% SDS, and heated to 90°C for 5 min. The lysate was then diluted with 9 vol of immunoprecipitation washing buffer containing 0.3% Triton X-100 and loaded on an anti-phosphotyrosine (PY20) column. The column was washed with immunoprecipitation washing buffer, and tyrosine-phosphorylated proteins were eluted in washing buffer containing 50 mM p-nitrophenylphosphate (Sigma). The eluate was concentrated in a Centricon concentrator (Amicon), subjected to SDS–PAGE, and transferred onto nitrocellulose. A single protein of 115 kDa could be stained on the membrane with amidoblack. The band was excised from the membrane and used for microsequencing.

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